Mycopathologia **157:** 333–338, 2004.

Expression of pectinase activity among *Aspergillus flavus* isolates from southwestern and southeastern United States

Jay E. Mellon & Peter J. Cotty
USDA, ARS, Southern Regional Research Center, P.O. Box 19687, New Orleans, Louisiana 70179, USA

Received 25 September 2002; accepted 16 July 2003

Abstract

Aspergillus flavus is a widely distributed filamentous fungus that contaminates crops with the potent carcinogen aflatoxin. This species can be divided into S and L strains on the basis of sclerotial morphology. During crop infection, *A. flavus* can secrete a large array of hydrolytic enzymes. These include pectinase, which aids fungal spread through plant tissues. A survey of pectinase expression by soil isolates derived from different regions of the United States revealed geographic polymorphisms. Strain L isolates from Arizona produced moderate to high levels of a specific pectinase P2c, while S strain isolates produced variable amounts of P2c. In contrast, L strain isolates from southeastern U.S. yielded variable P2c production, while S strain isolates consistently expressed high P2c levels. These results were corroborated by pectinase surveys of additional collections of *A. flavus* from soil and cottonseed. Expression patterns for P2c and pectinmethylesterase were evaluated for a select number of isolates using an isoelectric focusing technique. Clear zone reactions from the pectinase plate assay corresponded to the presence of P2c, while red ring reactions corresponded to the lack of P2c. Commercial cottonseed infected by S strain isolates frequently contained aflatoxin, even when infected by S strain isolates that did not produce pectinase P2c. Thus, although P2c-lacking isolates have reduced invasiveness, these isolates still have sufficient pathogenicity to cause aflatoxin contamination.

Key words: Aflatoxin, *Aspergillus flavus*, pectinase, pectinmethylesterase, polygalacturonase

Abbreviations: IEF, isoelectric focusing; PME, pectinmethylesterase

Introduction

The filamentous fungus *Aspergillus flavus* is a common saprophyte that is capable of opportunistic pathogenesis in plants and animals. The fungus has a negative economic impact on oilseed crops (corn, cotton, peanuts, tree nuts) through contamination with the potent carcinogen/mutagen aflatoxin B_1. The fungus produces a diverse range of polymer hydrolyzing enzymes, such as pectinases [1] and proteinases [2] to assist in the degradation of complex substrates.

Pectinases are thought to play important roles in plant pathogenesis by promoting tissue maceration [3]. The ability of *A. flavus* isolates to spread between locules of developing cotton bolls (fruits) is strongly correlated with the production of a specific endopolygalacturonase [4]. Moreover, this specific pectinase (P2c) is not catabolite repressed in culture by low molecular weight saccharides present in developing cotton bolls [5]. Further evidence for the importance of P2c in *A. flavus* invasiveness of cotton bolls is provided by the transformation of *A. flavus* strains lacking P2c with *pecA*, the gene which encodes this pectinase. Such transformants acquire an increased ability to cause intercarpellary membrane damage and invade adjacent locules [6]. In addition, removal of *pecA* activity through targeted disruption significantly reduces boll invasiveness [6].

A survey of 87 isolates of *A. flavus* from cottonseed and soil collected in southwestern Arizona demonstrated a variable distribution of pectinase production [1]. This collection of isolates consisted of two physiologically and morphologically distinct strains, S and L [7]. Strain S isolates produce abundant small sclerotia (<400 μm in diameter) and high levels of aflatoxin, while strain L isolates produce fewer but larger sclerotia (>400 μm in diameter), on average, and lower aflatoxin levels. All of the Arizona L strain isolates produced P2c, while 50% of the S strain isolates lacked P2c production [1]. Communities of *A. flavus* isolates from the Zhejiang province in southeast China differed from the Arizona *A. flavus* communities. The Zhejiang collection had no S strain isolates [8]. And, in contrast to Arizona *A. flavus* communities, L strain isolates from Zhejiang varied in pectinase production, with some L isolates failing to produce clear zones in the cup plate assay [8].

To test potential geographic variability among *A. flavus* communities, a survey of *A. flavus* resident in different regions of the Southern U.S. was conducted. Patterns of pectinase production were determined using a culture plate method [1]. For a select number of isolates, relative abundance of pectinase P2c and PME was determined with an electrophoretic technique [1, 5]. The results of this investigation are reported in this paper.

Materials and methods

Biological materials

Aspergillus flavus communities resident in agricultural soils were sampled by a dilution plate technique in southwestern (Arizona) and southeastern (Alabama, Arkansas, Louisiana, Mississippi) regions of the United States as previously described [9]. Specifics of soil sampling, including sample locations, sample size and soil depth, have been described [9]. Soil samples were collected in fields planted in cotton in a period from April to May of a given year. The isolates tested in Survey 1 (Table 1) were obtained in an earlier study [7]. The isolates tested in Survey 2 were obtained in a second soil sampling done in 1993 [9]. Soil samples were transported at ambient temperature to laboratory facilities and fungal isolations were accomplished in a timely manner (within a week from soil sampling). For each location sampled, a total of 20 to 35 isolates were obtained from 5 to 10 samples of soil [9]. Soil was suspended in water and spread on plates containing modified rose Bengal medium [10]. In order to compare fungal communities on cottonseed crops, seed samples (5–10 lb.) were requested from oil mills in each region (Survey 3, Table 1). The oil mills from which cottonseed samples were obtained were located as follow: Arizona, Casa Grande and Phoenix; Texas, LaMesa and Harlingen; Alabama/Georgia, Montgomery (AL), Macon (GA) and Albany (GA); Arkansas, Pine Bluff; Mississippi, Leland; Tennessee, Memphis. For cottonseed isolations, 1 to 30 isolates were obtained per location. Fungal propagules were washed from intact seed surfaces by manually shaking in 0.01% Triton X-100. Propagule suspensions were then spread on modified rose Bengal medium. Isolation plates were incubated for 3 days at 31 °C, after which discrete colonies were transferred to 5/2 agar (5% V-8 vegetable juice, 2% agar) [7]. Isolates were maintained on 5/2 agar at 30 °C or on agar plugs submerged in sterile deionized water [7]. Conidial suspensions in sterile deionized water (10^5 to 10^6 spores/ml) were used for inoculations.

Pectinase surveys

A total of 301 isolates from soil and cottonseed were assayed for polygalacturonase activity using a pectin-containing agarose medium [1]. Culture plates with pectin-containing agarose were inoculated with each isolate and incubated for 3 to 5 days at 31 °C. Isolates were replicated 2 to 3 times and tests were performed twice. After incubation, plates were stained with aqueous 0.05% ruthenium red solution for 1 h and rinsed with deionized water. Cultures expressing pectinase activity exhibited a clear zone (pectin digestion zone) around the margins of the colony. Isolates without a clear zone usually exhibited a ring of intense staining (red ring) around the colony. This zone was stained considerably darker than the background of the agar distant from the colony.

Pectinase production in liquid medium

In order to corroborate the production (or lack of) of pectinase P2c, selected isolates of *A. flavus* were grown in 70 ml of a liquid medium with the same composition as the survey medium, but lacking agarose [1]. Culture flasks (250-ml) were inoculated with 200 μl of spore suspension and agitated for 5 days at 32 °C (dark). Culture medium was separated from mycelial material by filtration *in vacuo*.

Electrophoresis

Samples for electrophoresis were prepared by concentrating culture filtrates 10- to 20-fold in centrifuge concentrators (Centricon, mol. wt. cutoff, 10 kDa; Amicon). Samples were stored at 4 °C until use. IEF gels were performed as previously described [11], except commercial 3.5–6.5 pH gradient gels (LKB) were used. Gel regions in contact with the electrolyte strips were removed; the gel was overlayered to a thickness of 2 to 3 mm with a pectin medium containing 0.5% pectin, 1.0% agarose, 50 mM potassium acetate (pH 5.2), and 10 mM EDTA. The gel/overlayer sandwich was incubated at 37 °C for 30 to 40 min, after which the gel was peeled off. The overlayer was stained with aqueous 0.1% ruthenium red solution for 12 min and destained with deionized water. Destained gel overlays were photographed to record data. Pectinase P2c [12] zones appeared as white bands, while PME bands stained dark red (black in photograph). The pI values were interpolated from gel pH gradient plots obtained by direct measurement with a surface pH electrode.

Individual cotton seed assays

In order to determine if failure to produce pectinase P2c influences the incidence and severity of aflatoxin contamination of cottonseed, cotton seeds exhibiting bright green-yellow fluorescence [13, 14] were collected from commercial gins (Arizona) and subjected to a split seed assay. Cottonseeds were surface sterilized for 1 min with 70% ethanol and rinsed with sterile deionized water for 30 sec. Each seed was bisected with a sterile razor blade. One seed half was placed on 5/2 agar and incubated at 30 °C to recover *A. flavus* isolates. Isolates belonging to the S strain were subjected to the pectinase culture assay. The seed half not used for fungal isolation was immersed in 2.0 ml of methylene chloride for 24 h to extract aflatoxin. Extracts were concentrated or diluted and analyzed for aflatoxins by thin layer chromatography. Extracts were spotted along side of aflatoxin standards (B_1, B_2, G_1, G_2; Sigma Chemical Co.) on silica gel plates and developed in diethyl ether/methanol/water (96 : 3 : 1) mobile phase. Aflatoxin B_1 was quantified directly on thin layer plates by fluorescence densitometry [15].

Results

The initial survey of *A. flavus* communities resident in agricultural soils demonstrated a clear dichotomy in pectinase expression between those obtained in the southwestern United States and those resident in the southeastern United States (Table 1A). The southwestern isolates of the L strain all produced a clear zone reaction in the pectinase culture assay, whereas 43% of southwestern S strain isolates produced no clear zone and exhibited a red ring reaction. S strain isolates were rare in Alabama; all southeastern S strain isolates produced clear zone reactions in the culture assay. However, 25% of southeastern L strain isolates produced red ring reactions (Table 1A).

Pectinase culture assay results for isolates collected during the second survey of *A. flavus* communities resident in Arizona and Alabama soils were very similar to those seen in the initial survey (Table 1B). To further extend the pectinase survey data obtained from soil isolates, a collection of *A. flavus* isolates was obtained from cottonseed. Communities of *A. flavus* present on cottonseed had a lower incidence of the S strain than those resident in soils. Therefore, relatively few S strain isolates from cottonseed were assayed for pectinase activity. However, of the twenty S strain isolates from cottonseed assayed, four exhibited a red ring reaction (Table 1C). These included all three isolates from Arizona and one from LaMesa, Texas. In a pattern similar to that seen with the soil communities, no S strain isolates from South Texas, Arkansas, Mississippi or Tennessee exhibited a red ring reaction, and no L strain isolate from Arizona yielded that reaction. But, 23% of L strain isolates from Alabama/Georgia did produce a red ring reaction (Table 1C). The pectinase survey data obtained from cottonseed isolates were consistent with the data derived from soil isolates.

To determine the expression patterns of pectinase P2c and PME, a select number of *A. flavus* isolates were subjected to analysis by native IEF gels. A typical gel is shown in Figure 1. This electrophoretic technique resolved both pectinase P2c and PME and allowed for a semiquantitative evaluation of isolate expression of these enzymes. The IEF data for selected *A. flavus* isolates is summarized in Table 2. Isolates that yielded clear zone reactions in the culture plate assay expressed moderate to high levels of pectinase P2c in liquid culture. Isolates exhibiting a red ring reaction expressed little or non-detectable levels of P2c in liquid culture. One isolate (MR3-15) that yielded a red ring reaction produced moderate P2c levels, but also produced high levels of PME. Another isolate (NG2-19) displaying a red ring reaction did not produce either P2c or PME, at least at the level of detection afforded by this IEF technique. In general, results of isolate analysis by IEF corresponded well with pectinase plate results.

In order to determine if pectinase expression patterns by *A. flavus* isolates (Arizona S strain) correlated

Table 1. Survey of *A. flavus* isolates for pectinase activity

Location	Substrate	# Isolates	Strain[a]	CZ[b]	RR[c]
A. Survey 1					
Southwest(AZ)	Soil	21	S	57	43
Southwest(AZ)	Soil	57	L	100	0
Southeast[d]	Soil	13	S	100	0
Southeast[d]	Soil	36	L	75	25
B. Survey 2					
Southwest(AZ)	Soil	28	S	50	50
Southwest(AZ)	Soil	29	L	100	0
Southeast(AL)	Soil	1	S	100	0
Southeast(AL)	Soil	33	L	55	45
C. Survey 3					
Southwest(AZ)	Cottonseed	3	S	0	100
Southwest(AZ)	Cottonseed	32	L	100	0
North Texas (La Mesa)	Cottonseed	1	S	0	100
South Texas (Harlingen)	Cottonseed	9	S	100	0
Southeast[e]	Cottonseed	7	S	100	0
Southeast[f]	Cottonseed	30	L	77	23

[a] Strain classification of *A. flavus* isolate [7].
[b] Percent of isolates displaying a clear zone reaction in pectinase culture assay.
[c] Percent of isolates displaying a red ring reaction in pectinase culture assay.
[d] Isolates obtained from Louisiana, Mississippi, and Alabama.
[e] Isolates obtained from Arkansas, Mississippi, and Tennessee.
[f] Isolates obtained from Alabama and Georgia.

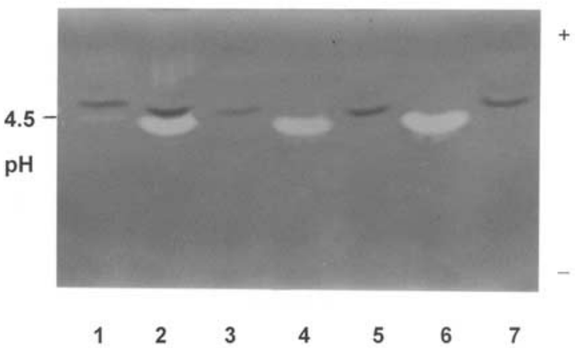

Figure 1. Pectinase P2c/PME profiles of *A. flavus* isolate culture filtrates. Culture filtrates were concentrated 10-fold before analysis; 20 μl of each sample was placed on the IEF gel. The polarity orientation of the gel is noted in the right margin and the pH is indicated in the left margin. Pectinase P2c activity appears as white bands, while PME activity appears as dark bands. Lane 1: AL1-3; lane 2: YV4-26; lane 3: W1-10; lane 4: MR5-31; lane 5: AL3-43; lane 6: AL1-1; lane 7: STV4-5.

Table 2. Electrophoretic survey of *A. flavus* isolates for pectinase P2c and PME activities

Isolate	Location	Strain[a]	Pectinase type[b]	P2c[c]	PME[c]
Pima-11	Arizona	L	CZ	+	−
MR2-15	Arizona	S	CZ	+ + +	−
D1-24	Arizona	S	RR	−	+
Wheat-4	Arizona	S	RR	−	++
D1-35	Arizona	S	CZ	+ + +	++
Pima-2	Arizona	S	RR	±	+
YV1-11	Arizona	S	CZ	+ + +	++
YV4-39	Arizona	S	RR	−	+
MR2-7	Arizona	L	CZ	+ + +	+
MR3-15	Arizona	S	RR	++	+ + +
NG1-4	Arizona	S	CZ	++	−
YV4-26	Arizona	S	CZ	+ + +	++
W1-10	Arizona	S	RR	−	±
MR5-31	Arizona	S	CZ	+ + +	−
NG2-19	Arizona	S	RR	−	−
Miss2-19	Louisiana	L	CZ	+ + +	−
STV35-29	Mississippi	S	CZ	+ + +	−
STV4-5	Mississippi	L	RR	−	+
AL1-42	Alabama	L	CZ	++	−
AL3-22	Alabama	S	CZ	+ + +	−
AL1-25	Alabama	L	RR	−	+
AL2-22	Alabama	L	RR	−	+
AL1-1	Alabama	L	CZ	+ + +	−
AL1-20	Alabama	L	RR	−	−
AL2-26	Alabama	L	CZ	+ + +	−
AL2-32	Alabama	L	RR	±	±
AL3-5	Alabama	L	RR	±	+
AL1-3	Alabama	L	RR	±	+
AL3-43	Alabama	L	RR	−	+

[a] Strain classification of *A. flavus* isolate [7].
[b] Reaction type derived from pectinase culture assay: CZ = clear zone; RR = red ring.
[c] Abundance of pectinase P2c and PME activities on a scale of − to + + + where − indicates lack of activity and + + + indicates maximal activity; ± indicates trace levels.

with aflatoxin contamination levels in cottonseed, a series of S strain isolates from individual seeds was assayed for aflatoxin B_1 and pectinase production. There was no apparent correlation between aflatoxin levels within the infected seed and pectinase production by the *A. flavus* isolated from that seed (Table 3). Seed that was highly contaminated with aflatoxin yielded fungal isolates that either did or did not display pectinase P2c production. In addition, seed that was not aflatoxin contaminated also yielded *A. flavus* isolates (S strain) that did or did not produce P2c.

Table 3. Aflatoxin levels and pectinase type of *A. flavus* isolates (S strain) derived from individual cotton seeds

Year	# Isolates	Pectinase type[a]	Aflatoxin B_1 (ppb)[b] Mean	Range
1990	6	RR	3.14×10^6	$0–6.82 \times 10^6$
	5	CZ	1.91×10^6	$0–5.95 \times 10^6$
1991	2	RR	1.86×10^5	$0–3.71 \times 10^5$
	5	CZ	8.30×10^5	$0–2.89 \times 10^6$
1993	13	RR	4.33×10^3	$0–5.15 \times 10^4$
	11	CZ	1.13×10^5	$0–1.08 \times 10^6$

[a] Reaction type derived from pectinase culture assay: CZ = clear zone; RR = red ring.
[b] Aflatoxin levels for seed infected by RR and CZ isolates do not differ significantly (ANOVA, $P > 0.05$) for any year.

Discussion

The initial survey of *A. flavus* soil isolates suggested a geographical polymorphism in pectinase production among S and L strains of the fungus derived from different regions of the Southern U.S. This observation was confirmed by the second pectinase screening performed on a collection of *A. flavus* soil isolates derived from a re-sampling of locations in Arizona and Alabama. The pectinase screen (culture assay) of *A. flavus* isolates derived from cottonseed further strengthened these results. These three sets of results were consistent. Strain L isolates from the southwestern U.S. (Arizona) produced pectinase P2c (clear zone reaction), while strain S isolates demonstrated variable production of P2c. This result is consistent with a previous report [1]. In contrast, strain L isolates from southeastern U.S. showed variable P2c production, while strain S isolates consistently yielded P2c activity.

Variability of various characters, including aflatoxin/pectinase production and sclerotial size, among *A. flavus* isolates has been reported for regions of China [8]. The data reported here support a similar geographical variability among *A. flavus* isolates in pectinase production throughout southwestern and southeastern regions of the U.S. Pectinase P2c of *A. flavus* is linked to fungal invasiveness and the ability to infect adjacent locules of developing cotton bolls [4, 5]. Isolates that produce high levels of P2c may be better adapted to reproduce and survive on plant material, and it has been suggested that S strain isolates that lack P2c production may be adapted to certain soil niches where the selective advantage in producing pectinase is reduced or lacking [16]. In contrast to observations for *A. flavus* isolates from Arizona, strain L isolates from the Southeastern U.S. demonstrated variable P2c production. This may result from L strain isolates resident in the Southeast U.S. being adapted to a niche where pectinase is not essential. It is interesting to note that S strain isolates are relatively rare in the *A. flavus* populations sampled in Alabama (current report, [9]). This observation may reflect differences in ecological niches to which *A. flavus* is adapted in the two regions.

There are considerable differences between the Southwestern and Southeastern United States with respect to climate and crop composition. The southwestern region generally receives lower annual rainfall (<25 cm/year) and displays low humidity (0 to 40% relative humidity). Thus, most crop production requires irrigation. Southeastern U.S regions, on the other hand, receive greater annual rainfall levels, on average, and display higher humidity levels. Although native plant species vary widely across these regions, cotton is a major crop in all areas studied. All these factors contribute to differences in ecological niches to which *A. flavus* has adapted.

The IEF data confirmed that a red ring reaction in the pectinase culture assay resulted from lack of production of pectinase P2c, while a clear zone response resulted from moderate to high levels of P2c production. *Aspergillus flavus* isolates also displayed variability in PME production. However, PME expression was not correlated with pectinase reaction type (plate assay) or any other known isolate characteristic. The pI values for P2c and PME obtained from this study agreed with those of an earlier report [5], corroborating the identity of these enzyme activities.

A. flavus S strain isolates that produce nondetectable quantities of P2c have been shown to have reduced capacity to both decay developing cotton bolls and spread between cotton locules [4, 7]. Increasing P2c expression by transforming such *A. flavus* strains with *PecA*, the gene coding for P2c, results in increased virulence [6]. The current study shows, for the first time, that isolates expressing either reduced or no P2c still can cause significant aflatoxin contamination in the commercial crop. In greenhouse tests, reduced virulence associated with lack of P2c resulted in reduced contamination of developing wound-inoculated bolls [4, 7]. However, because S strain isolates are highly toxigenic, bolls inoculated with P2c-lacking isolates still became significantly contaminated [7].

In the current study, failure to see differences between the aflatoxin levels of seed infected with either P2c-producing or P2c-lacking S strain isolates probably reflects the importance of environmental factors in determining the extent of contamination. Variation in both time of infection and environmental conditions (i.e., humidity and temperature) to which individual seed are exposed are likely causes of the very wide ranges of observed contamination. Environment apparently has a greater influence on aflatoxin contamination than the natural variation in isolate virulence observed in *A. flavus* communities.

Acknowledgments

The technical assistance of R. Roberson and D. Downey is greatly appreciated.

References

1. Cotty PJ, Cleveland TE, Brown RL, Mellon JE. Variation in polygalacturonase production among *Aspergillus flavus* isolates. Appl Environ Microbiol 1990; 56: 3885–3887.
2. Mellon JE, Cotty PJ. Expression of elastinolytic activity among isolates in *Aspergillus* section *Flavi*. Mycopathologia 1995; 131: 115–120.
3. Collmer A, Keen NT. The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 1986; 24: 383–409.
4. Cleveland TE, Cotty PJ. Invasiveness of *Aspergillus flavus* isolates in wounded cotton bolls is associated with production of a specific fungal polygalacturonase. Phytopathology 1991; 81: 155–158.
5. Brown RL, Cleveland TE, Cotty PJ, Mellon JE. Spread of *Aspergillus flavus* in cotton bolls, decay of intercarpellary membranes, and production of fungal pectinases. Phytopathology 1992; 82: 462–467.
6. Shieh MT, Brown RL, Whitehead MP, Cary JW, Cotty PJ, Cleveland TE, Dean RA. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl Environ Microbiol 1997; 63: 3548–3552.
7. Cotty PJ. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 1989; 79: 808–814.
8. Wang ZG, Tong Z, Cheng SY, Cong LM. Study on pectinase and sclerotium producing abilities of two kinds of *Aspergillus flavus* isolates from Zhejiang. Mycopathologia 1993; 121: 163–168.
9. Cotty PJ. Aflatoxin-producing potential of communities of *Aspergillus* section *Flavi* from cotton producing areas in the United States. Mycol Res 1997; 101: 698–704.
10. Cotty PJ. Comparison of four media for the isolation of *Aspergillus flavus* group fungi. Mycopathologia 1994; 125: 157–162.
11. Mellon JE, Lee LS. Elicitation of cotton isoperoxidases by *Aspergillus flavus* and other fungi pathogenic to cotton. Physiol Plant Pathol 1985; 27: 281–288.
12. Cleveland TE, McCormick SP. Identification of pectinases produced in cotton bolls infected with *Aspergillus flavus*. Phytopathology 1987; 77: 1498–1503.
13. Ashworth LJ Jr, McMeans JL. Association of *Aspergillus flavus* and aflatoxins with a greenish yellow fluorescence of cotton seed. Phytopathology 1966; 56: 1104–1105.
14. Lee LS, Cucullu AF, Pons WA Jr. Separation of aflatoxin-contaminated cotton seed based on physical characteristics of seed cotton and ginned seed. J Am Oil Chem Soc 1977; 54: 238A–241A.
15. Stoloff L, Scott PM. Natural poisons. In: Williams S, ed. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed. Arlington, VA: Association of Official Analytical Chemists, Inc., 1984, 477.
16. Cotty PJ, Bayman P, Egel DS, Elias KS. Agriculture, aflatoxins and *Aspergillus*. In: Powell KA et al., eds. The Genus *Aspergillus*. New York: Plenum Press, 1994; 1–27.

Address for correspondence: J. E. Mellon, USDA, ARS, SRRC, P.O. Box 19687, New Orleans, LA 70179, USA
Phone: 1-504-286-4358; Fax: 1-504-286-4419;
E-mail: jmellon@srrc.ars.usda.gov